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Tylopilus: a new species and a new record from Pakistan

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Abstract — *Tylopilus sultanii* sp. nov. and *T. pseudoscaber* are reported from Pakistan, occurring in forests dominated by ectomycorrhizal trees of *Pinaceae*. The collections are described and illustrated, and their habitat is described. Their phylogenetic relationships were accessed by ITS sequence analysis. *Tylopilus pseudoscaber* represents a new record for Pakistan. A key to the three *Tylopilus* species recorded in Pakistan is presented.

Key words — *Basidiomycota*, biogeography, *Boletaceae*, conifers, Himalaya, phylogeny

Introduction

Khyber Pakhtunkhwa (KPK), Pakistan, which lies at an altitude of 1000–4000 m in the Himalayan range, is mainly dominated by conifer forests and regarded as one of the world's 35 biodiversity hotspots (Myers et al. 2000, Zachos & Christian 2011). The Mansehra, Dir, Swat, Malakand, and Abbottabad districts of KPK, and the Rawalpindi district of the Punjab are covered mainly by conifer forests (Anwar 2008), including fir (*Abies*), deodar (*Cedrus deodara*), Kail (*Pinus wallichiana*), and chir pine (*Pinus roxburghii*). Normal growth and survival of pinaceous trees depends on colonization by ectomycorrhizal (ECM) fungi (Smith & Read 1997).

The ectomycorrhizal genus *Tylopilus* P. Karst. has a rich biogeographical history (Wolfe & Bougher 1993, Halling 1996) and is characterized by smooth spores, a pinkish to black pore surface, solid stipe lacking an annulus, and glandular dots (Bessette et al. 2000). Seventy-six *Tylopilus* species are known worldwide (Kirk et al. 2008, Osmundson & Halling 2010), but molecular analysis shows that the genus is polyphyletic (Binder & Hibbett 2006).

Only one *Tylopilus* species, *T. felleus* (Bull.) P. Karst., has been reported from Pakistan (Razaq 2007). We describe here a new species, *T. sultanii,* and a new record for the country, *T. pseudoscaber*. A key to the three *Tylopilus* species in Pakistan is also provided.

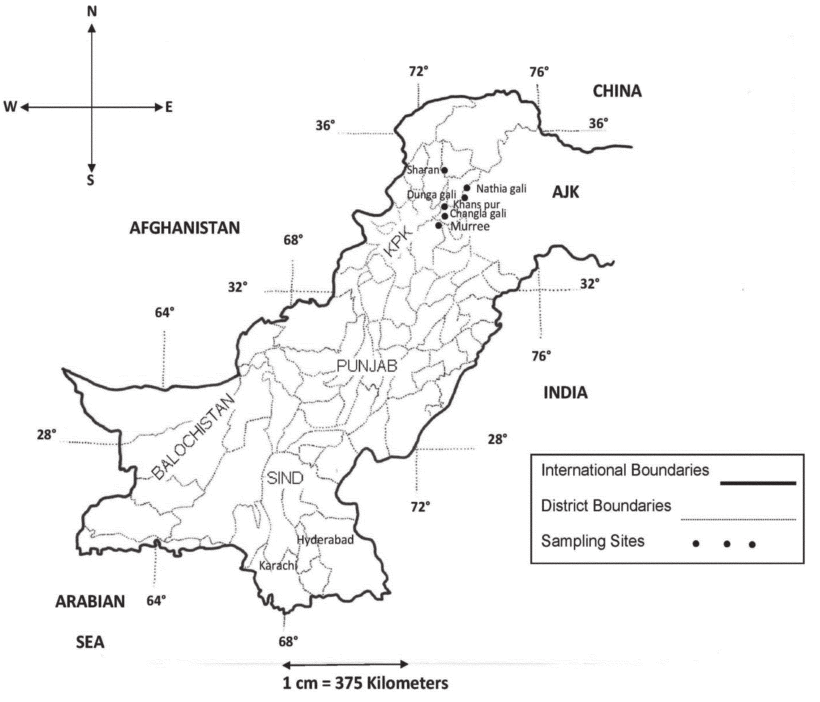

Figure 1. Map of Pakistan showing sampling sites.

Materials & methods

Sporocarp collection

Specimens were collected from sampling sites (Fig. 1) in early to late summer (July–September) during 2006, 2009, and 2011. Field notes and digital photographs were made from fresh specimens. Sporocarps were dried with hot air from a fan heater and then kept in paper bags for processing in the lab. Voucher specimens were deposited in the bolete collection of the Lahore Herbarium, Department of Botany, University of the Punjab, Lahore, Pakistan (LAH).

Morphological characterization

Characters recorded from fresh sporocarps included:

Pileus: Color, shape, diameter, texture and ornamentation, context color and color changes, margin shape and color.

Hymenophore: young and mature tube and pore color, tube and pore size, and bruising reactions of the pore surface.

Stipe: Color, shape, diameter, texture and ornamentation, context color and color changes, presence/absence of annulus, attachment to the pileus.

Characters recorded after mounting dried sporocarp tissue in KOH, Meltzer's reagent, Trypan blue, and Lactic acid included:

> Shape, length, width and cytoplasmic contents of basidia, cystidia, basidiospores, pileipellis and terminal cells of pileipellis; color reaction of all these in KOH, Meltzer's, lactic acid and Trypan blue.

Molecular analysis

DNA was extracted from dried sporocarps by using enzymatic digestion and glass-fibre filtration (EDGF) protocol (Dentinger et al. 2010). The nuclear ribosomal internal transcribed spacer region (ITS) was amplified following the PCR conditions of Dentinger et al. (2010) using primers ITS3 (White et al. 1990) and ITS6R (Dentinger et al. 2010). PCR products were purified using ExoSAP-IT® (Affymetrix, High Wycombe, UK) and dye-terminated unidirectional sequencing was performed using a BigDye®Terminator v3.1Cycle Sequencing Kit (Life Technologies/ABI, California, USA) in 10μL reactions with primers ITS3 and ITS6R following the protocol of Dentinger et al. (2010). Sequencing reactions were cleaned using ethanol precipitation following the manufacturer's instructions, resuspended in 30 μL of distilled water, and run on an ABI 3730 DNA sequencer in the Jodrell Laboratory, Royal Botanic Gardens Kew. Sequence chromatograms were edited by comparing overlapping reads using BioEdit and comparisons to GenBank records using BLAST. Sequences have been deposited in a single file in GenBank (*Tylopilus pseudoscaber:* KJ775785; *T. sultanii:* KJ775786). The sequences were aligned using MUSCLE alignment software (Edgar 2004). Phylogenetic trees were constructed with the maximum likelihood algorithm and Jukes & Cantor (1969) model of sequences evolution using Model-testing feature of MEGA5 software (Tamura et al. 2011). A bootstrap consensus tree (Fig. 5) was inferred from 1000 replicates, and corresponding bootstrap values >50% are cited in the tree.

Taxonomy

Tylopilus sultanii S. Sarwar, Khalid & Niazi, **sp. nov.** Figs 2–3

MycoBank MB 802339

> Differs from *Tylopilus pseudoscaber* by its context that does not change color when bruised, its larger basidiospores, and its cracked pileus surface.
>
> Type: Pakistan, Khyber Pakhtunkhwa, Ayubia, 2350 m a.s.l., under *Pinus wallichiana* A.B. Jacks., on ground, 15 August 2006, A.R. Niazi 36 (**Holotype**, LAH0806; GenBank KJ775786).
>
> Etymology: referring to Dr. Sultan Ahmad, a pioneer mycologist in Pakistan.

Pileus 6–9 cm wide, globose, hemispherical to broadly convex, dark brown, surface dry, rough, cracked into rectangular patches, context pinkish yellow to creamy visible between patches, margins straight to deflexed, same color as pileus surface, even margins. Context creamy, no color change upon exposure. Odor and taste not distinctive. Stipe about 5 cm long, 3 cm thick, central, solid, hard, smooth, slightly curved, the center of stipe dark brown to black, at top and base whitish to creamy in color, context like pileus, no color change upon

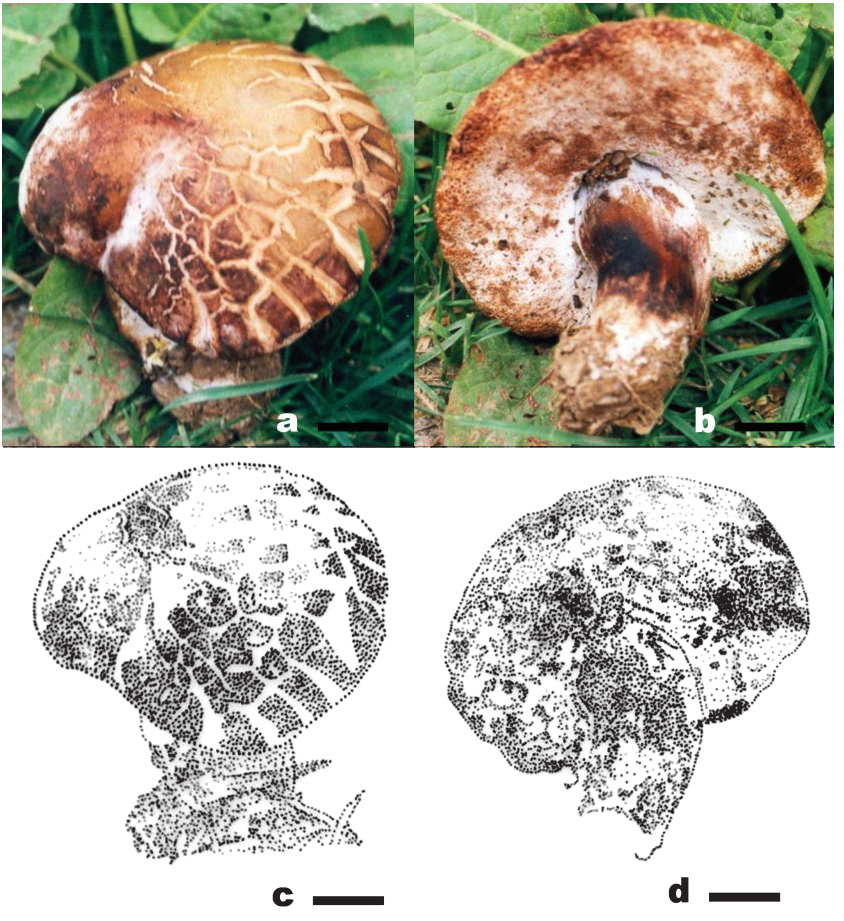

Figure 2. *Tylopilus sultanii* (holotype, LAH0806).
a, b. Sporocarps, in situ. c,d. Sporocarps. Scale bars = 1.5 cm.

exposure. Pore surface whitish brown to dark brown, adnate and horizontal to sinuate, pores 2–3 per mm, tubes whitish to off-white, 9–17 mm deep, no color change to slightly brown upon bruising. Edibility not known.

Basidiospores oblong–inequilateral, apiculate, smooth, 18–19 × 8–10 µm, (18.4 ± 0.45 × 9.13 ± 0.74; Q_m = 2.07 ± 0.18). Basidia clavate, 2–4-sterigmate, sterigmata long, contents visible, brownish pigments visible, 62–64 × 19–20 µm. Cystidia cylindrical to subclavate, thin walled, hyaline, 32–35 × 9–10 µm. Pileipellis a layer of long cylindrical septate hyphae, 76–80(–97) × 10–12 µm.

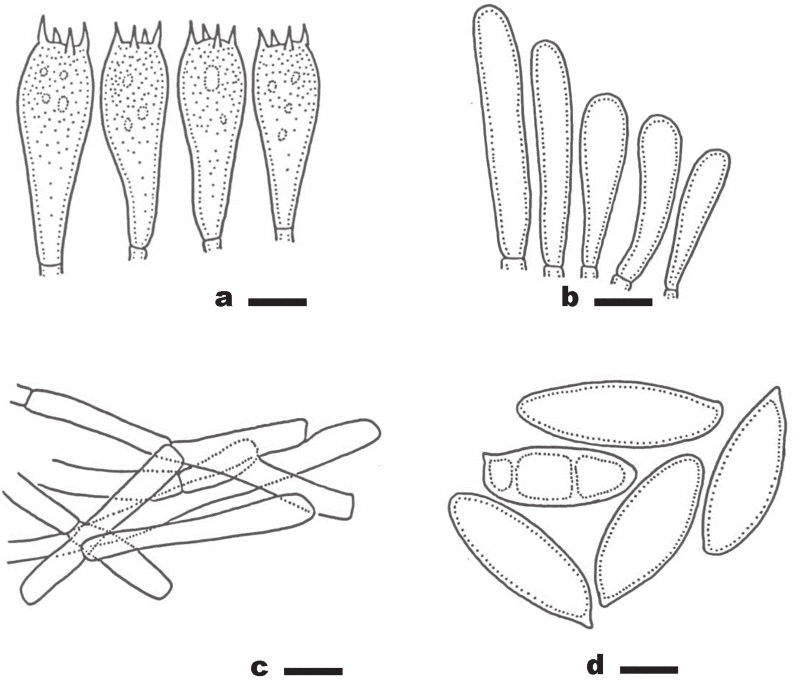

Fig. 3. *Tylopilus sultanii* (holotype, LAH0806).
a. Basidia. b. Cystidia. **c**. Pileipellis hyphae. d. Basidiospores.
Scale bars: a = 16 μm; b = 9.5 μm; c = 17 μm; d = 6 μm.

Chemical reactions: pileipellis staining reddish brown in KOH; spores yellowish brown to reddish brown in Meltzer's reagent.

Ecology & distribution: Known only from the type locality, fruiting July–September during rainy season.

Notes: *Tylopilus sultanii* is similar to *T. badiceps* (Peck) A.H. Sm. & Thiers, *T. indecisus* (Peck) Murrill, and *T. pseudoscaber*. However, *T. badiceps* differs by its obliquely truncate pileus, stipe with a whitish apex, reticulations near the apex, and spores without an apiculus; *T. indecisus* differs by its pale brown to dull cinnamon pileus surface and reticulated stipe; and *T. pseudoscaber* differs by its context bluing upon bruising and its smaller basidiospores (Thiers 1975, Bessette et al. 2000, Lakhanpal 1996).

Tylopilus pseudoscaber (Singer) A.H. Sm. & Thiers, Mycologia 60: 950 (1968) Fig. 4
≡ *Porphyrellus pseudoscaber* Singer, Farlowia 2: 115 (1945)

Pileus 5–9 cm wide, convex to broadly convex, surface dry, smooth, dull, dark olive brown to dark brown, margins smooth, entire, same color as pileus, deflexed to slightly incurved. Context white to off–white, slowly bluing upon exposure, and then turning brownish. Stipe 6–11 cm long, 1.5–3 cm thick, subclavate to equal, straight to slightly curved, dry, dull, solid, centric, color like pileus surface, whitish near base, sometimes brownish dots present, context off-white, change to brownish when exposed. Pore surface dark reddish brown to blackish brown, staining dark blue at first then turning dark brown, pores 2–3/mm, circular to angular, narrow, 2–3 per mm, tubes 1–2.5 cm deep, shorter towards margins, brownish. Smell pungent, Taste not distinctive. Edibility not known.

Basidiospores ellipsoid to subfusoid, smooth, thick walled, 11–16 × 6–8 µm, (13.6 ± 1.6 × 7.07 ± 0.72; Q_m = 2.05 ± 0.37). Basidia clavate, 3–4 sterigmate, thick walled, 34–41 × 11–13 µm. Cystidia subclavate to fusoid to ventricose, with tapering tips, brownish in KOH, 39–59 × 11–17 µm. Pileipellis cylindrical interwoven hyphae, 52–58 × 5–8 µm; most terminal elements cylindrical with pointed tips in some cases, 43–50 × 7–9 µm.

Chemical reactions: pileipellis staining yellowish red in KOH, Meltzer's reagent; spores ochraceous in Meltzer's reagent, light yellow in lactic acid.

Ecology: In Pakistan, fruiting under coniferous trees, July–September during the rainy season.

Material examined: **PAKISTAN**, **Khyber Pakhtunkhwa**, Khaira gali, 2347 m a.s.l., solitary on ground under *Abies pindrow* Royle, 17 July 2009, Sarwar S.B. 20 (LAH0709: GenBank KJ775785); Nathiagali, 2439 m a.s.l., solitary on ground under *Pinus wallichiana* A.B. Jacks., 19 July 2009, Sarwar S.B. 20A (LAH0709); Sharan (Kaghan valley), 2011 m a.s.l., solitary on ground under *Pinus wallichiana* A.B. Jacks., 7 August 2011, Tayiba A. 68 (LAH0811).

Notes: *Tylopilus pseudoscaber* is similar to *T. porphyrosporus* (Fr.) A.H. Sm. & Thiers, *T. indecisus*, and *T. sordidus* (Frost) A.H. Sm. & Thiers. However, *T. porphyrosporus* differs by its larger size and its context not bluing upon bruising; *T. indecisus* differs by its pale brown to dull cinnamon pileus surface and reticulated stipe; and *T. sordidus* differs by its longitudinal streaks on the stipe (Bessette et al. 2000, Thiers 1975).

Key to *Tylopilus* species recorded from Pakistan

1. Stipe with brownish base, with reticulations ..... *T. felleus*
1. Stipe with whitish base, without reticulations 2
2. Basidiospores 11–16 × 6–8 µm; context bluing upon bruising ..... *T. pseudoscaber*
2. Basidiospores 18–19 × 8–10 µm; context not bluing upon bruising ..... *T. sultanii*

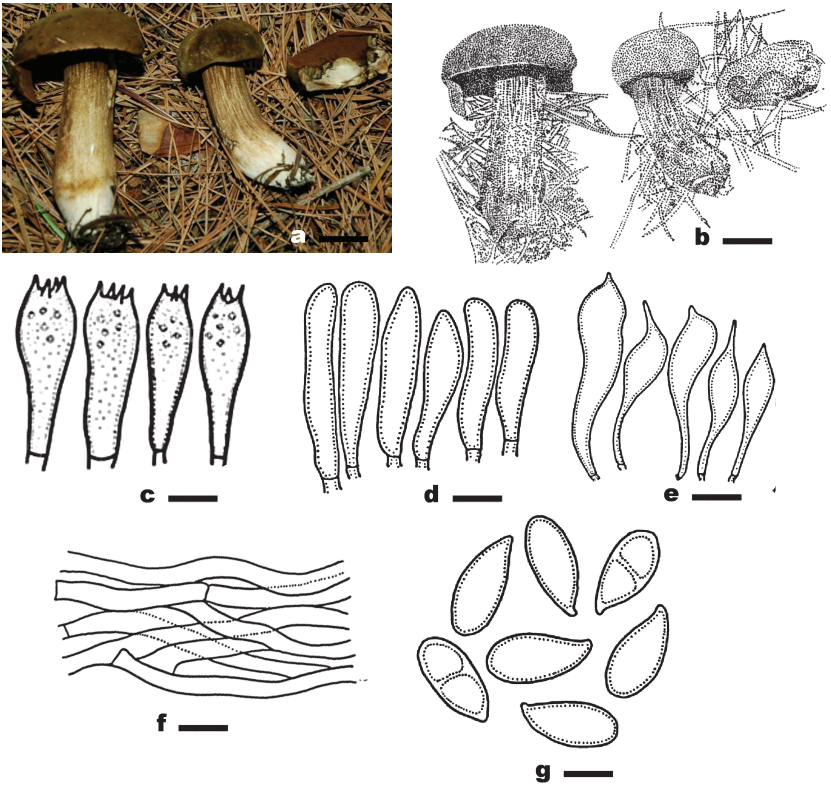

Figure 4. *Tylopilus pseudoscaber* (LAH0709).
a, b. Sporocarps. c. Basidia. d. Cystidia. e. Terminal elements of pileipellis hyphae.
f. Pileipellis hyphae. **g**. Basidiospores.
Scale bars: a, b = 3.5 cm; c = 8 µm; d, e = 13 µm; f = 14 µm; g = 7 µm.

Phylogenetic analysis

The closest BLAST matches for our two ITS sequences were *T. pseudoscaber* (JF899578) and *Porphyrellus pseudoscaber* (EU685112); our *T. pseudoscaber* (KJ775785) sequence showed 99% similarity with both of these, but our *T. sultanii* (KJ775786) sequence showed only <95% similarity.

The maximum likelihood phylogenetic tree (Fig. 5) had 674 genetic characters after alignment and trimming from both 3 and 5 sites of rDNA–ITS. No characters were excluded from the final analysis, all characters were of the 'unord' type, all gaps were treated as "missing" data, and multistate characters were interpreted as uncertain. The phylogram contained three clades; both of our sequences clustered in clade I.

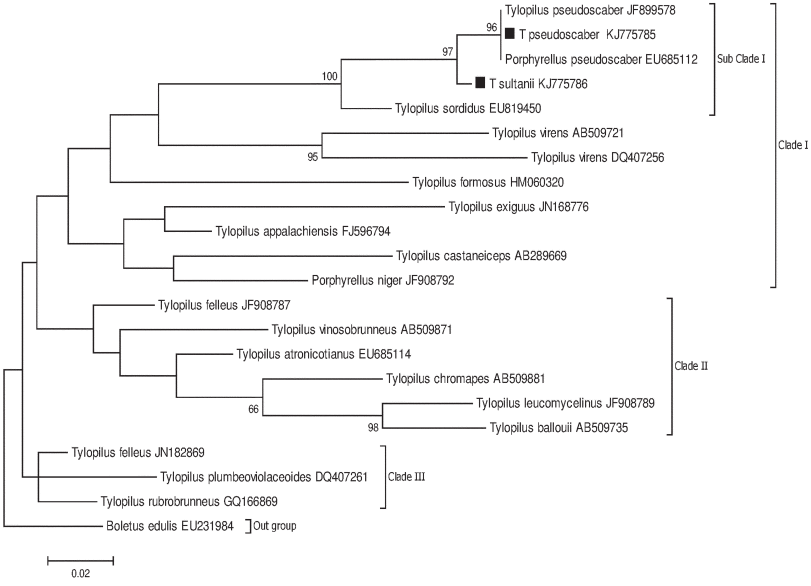

FIGURE 5. Phylogenetic position of *Tylopilus sultanii* and *T. pseudoscaber* from Pakistan relative to other *Tylopilus* spp., with *Boletus edulis* as outgroup. Tree inferred by maximum likelihood analysis based on 5.8S+ITS2 rDNA sequences. Numbers against branches indicate percentage support (>50%) in 1000 bootstrap replications. GenBank accession numbers stand after species names. ▪ = new sequences from Pakistan.

Tylopilus sultanii was shown to be phylogenetically closest to *Porphyrellus pseudoscaber* (EU685112), with which it shares 91.2% genetic characters while being separated by a 1.9% genetic divergence. There was significant genetic divergence of rDNA–ITS between *T. sultanii* and all other rDNA sequences included in the present study. Maximum similarity and shared genetic characters were well below the 97% cutoff value for species delimitation, and genetic divergences were high enough to confirm this species as new.

Our *T. pseudoscaber* sequence shared 99.9% genetic characters with both *T. pseudoscaber* (JF899578) and *Porphyrellus pseudoscaber* (EU685112) with only 0.2% genetic divergence.

Discussion

Both morphological and molecular analysis confirm *T. sultanii* as a new species, forming a highly supported sister clade to *T. pseudoscaber*.

Our sequence analysis confirmed our morphological identification of our Pakistani *T. pseudoscaber* collections, which formed a very highly supported clade with North American sequences of *T. pseudoscaber.* This is a new record for Pakistan.

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